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THE SEASONAL CYCLE OF COPPER CONCENTRATION IN *BUSYCON CANALICULATUM* L.

SUSAN B. BETZER[1] AND MICHAEL E. Q. PILSON

Graduate School of Oceanography, University of Rhode Island, Kingston, Rhode Island 02881

Copper is present in seawater in only trace amounts, on the order of 3 μg/l (Goldberg, 1965). Nevertheless, the copper concentration in marine gastropods has been shown to be generally high, up to 100 μg/g (Marks, 1938; Vinogradov, 1953; Segar, Collins and Riley, 1971). This implies the presence of a mechanism for storing copper taken up either from the diet or directly from seawater. Riley and Segar (1970) have stated that for marine animals in general, "Little is known about the mechanisms by which trace elements are concentrated or about the manner in which they are held in tissues" (page 721).

Marine gastropods are known to use copper (in the synthesis of the blood pigment, hemocyanin), yet for no species has there been a description of copper distribution in the body complete enough to allow inferences to be drawn about either the extent of individual variation or the physiological and ecological factors which control it. In the present study, specimens of the locally abundant channeled whelk, *Busycon canaliculatum* L., have been examined for copper concentration in various organs and tissues at different seasons of the year.

Materials and Methods

Approximately 100 specimens of *Busycon canaliculatum* L. were obtained from the region between Wickford and Fox Island in Narragansett Bay, Rhode Island, by collection in pots during the summer and fall and by dredging during the winter and spring and were held without feeding in running bay water until used. The weights of the animals used ranged in general from 100 to 300 g. Average tissue weight (total fresh weight minus shell weight) was 120 g.

Whelks were removed from the shell, and whole organs and tissue samples were dissected free and weighed. Blood samples were obtained by placing the body in a funnel, cutting into the foot muscle with a scalpel, and allowing blood from the pedal sinus to drain into a graduated centrifuge tube. Some samples of blood were obtained, prior to dissection, from a hole drilled in the operculum.

Blood samples were centrifuged and either analyzed immediately or frozen and stored for later analysis of protein and copper concentration. Protein was determined by the Biuret method (Layne, 1957) after 10- or 20-fold dilution with 0.1 M KCl. Bovine serum albumin was used as a protein standard.

Samples of blood and tissue were prepared for copper analysis by digesting with a solution made by mixing 100 ml of concentrated perchloric acid and 400 ml of concentrated nitric acid. The samples were placed in Pyrex® or Vycor® beakers or Kjeldahl flasks, with 5 ml or more of the acid solution, and heated gently until

[1] Present address: Department of Marine Science, University of South Florida, St. Petersburg, Florida 33701.

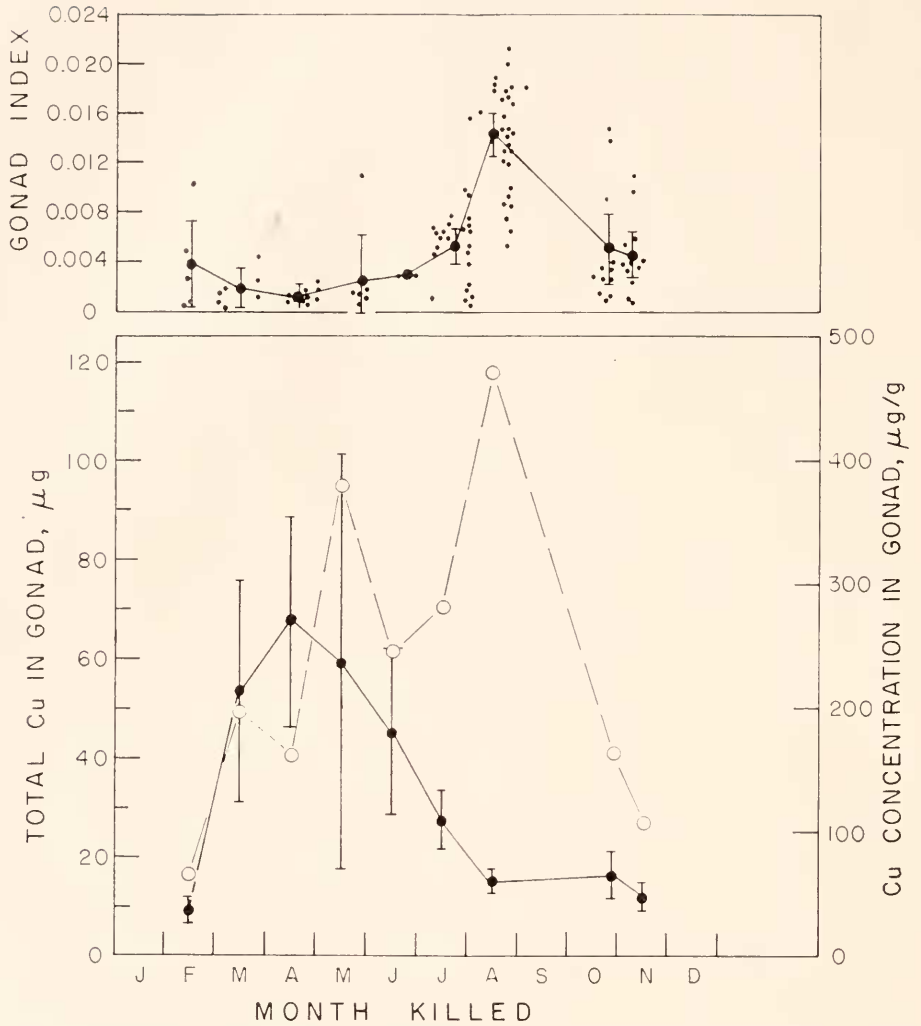

FIGURE 1. Annual changes in gonad index and gonad copper in *Buscycon*. Gonad index (fresh weight gonad/fresh weight whole soft tissues): points represent individual animals; closed circles, average gonad index for month; vertical bars represent two standard errors of the mean (S.E.M.) for month; open circles, monthly average total copper in gonad of 120-gram whelk (see text); closed circles, average copper concentration for month ± one S.E.M. Individual data on copper concentration which are summarized in Figures 1–4 are tabulated in Appendix IV of Betzer (1972).

all organic matter was oxidized and the nitric acid had boiled off. Ordinarily the digestive gland, gut, kidney, gonad, osphradium, and gill were each analyzed whole, while aliquots weighing 0.5–1.0 g were taken from other tissues (foot muscle, mantle, nidamental gland, oviduct or blood diluted with 0.1 M KCl). After digestion, the samples were analyzed either by atomic absorption spectroscopy or by the

spectrophotometric cuproine method (B) of Diehl and Smith (1958). For atomic absorption analysis the samples were rinsed into volumetric flasks and made up to volume with deionized water, to give, where possible, a final copper concentration of 1–10 μg/ml. The solutions were run against double-deionized water on a Perkin-Elmer Model 303 atomic absorption unit, using a laminar flow burner with an air-acetylene flame. Samples were alternated with copper standards and run in triplicate. In analyses by the cuproine method, if a sample was thought to contain less than about 25 μg of copper, the whole sample was used for the analysis; samples containing greater amounts of copper were diluted with deionized water in volumetric flasks, and duplicate aliquots containing 5–15 μg of copper were analyzed. The cuproine method was modified by the addition of 5 g/l of hydroquinone to the cuproine reagent to retard fading of color (Riley and Sinhaseni, 1958). In the cuproine procedure, copper is extracted from the aqueous phase by combination with cuproine dissolved in isoamyl alcohol. It was found that better separation of water droplets from the organic layer in the final centrifugation step was achieved if the alcohol-cuproine solution was refrigerated before centrifugation. Standards (prepared from copper shot dissolved in reagent grade concentrated nitric acid and diluted with deionized water) and blank samples were prepared and analyzed in the same way as the tissue samples.

The average standard deviation for triplicate copper determinations by atomic absorption spectroscopy was 2.6% of the determined values. Cuproine determinations were ordinarily done in duplicate, and the average standard deviation calculated from duplicate aliquots from the same solution was 0.70% of the determined values; the average standard deviation calculated from duplicate samples of blood oxidized with acid in separate Kjeldahl flasks and then analyzed was 1.05% of the determined values. The average recovery of standards boiled with acid and then analyzed was 104% of the values for standards analyzed directly by the cuproine method. When the same samples were analyzed by both atomic absorption and cuproine methods, the average difference between results by the two techniques was 6.8% of the mean copper concentration.

The total body copper was determined in 6 whelks. Each was placed over a large watch glass during dissection to catch any blood or mucous running from the shell. After whole organ and tissue samples had been taken, the remaining whelk tissues were washed into a Waring Blendor with a known quantity of deionized water. The blender was run until the mixture appeared homogeneous, and then while it was still running a dropper was used to transfer aliquots to two Vycor® or Vitreosil® crucibles. All the crucibles were weighed to determine fresh weight of the tissue and then were dried overnight at 100° C and reweighed. They were then covered, placed in a muffle furnace, and ashed at 550° C for 8–20 h. After cooling, 1–2 ml of concentrated HCl was added to each crucible and allowed to stand about 1 h. The contents of the crucibles were then transferred with deionized water rinsing to volumetric flasks, for copper determination by the cuproine method.

Results

Seasonal changes in copper concentrations in individual tissues

Busycon canaliculatum undergoes a seasonal cycle in the bay; during the warm summer months (beginning in late May or early June) whelks move about on

the sediment, actively feeding, and are caught commercially in baited "pots." During the colder months, from late fall until late spring, the whelks lie buried in the sediment, no longer attracted by bait. The reproductive cycle shows a similar seasonal pattern, as can be seen from the annual changes in the gonad index (fresh weight gonad/fresh weight whole soft tissues) (Fig. 1). From this graph it appears that spawning may occur primarily in the late summer and fall, although occasional ripe individuals (having a gonad index greater than 0.002–0.003 and a

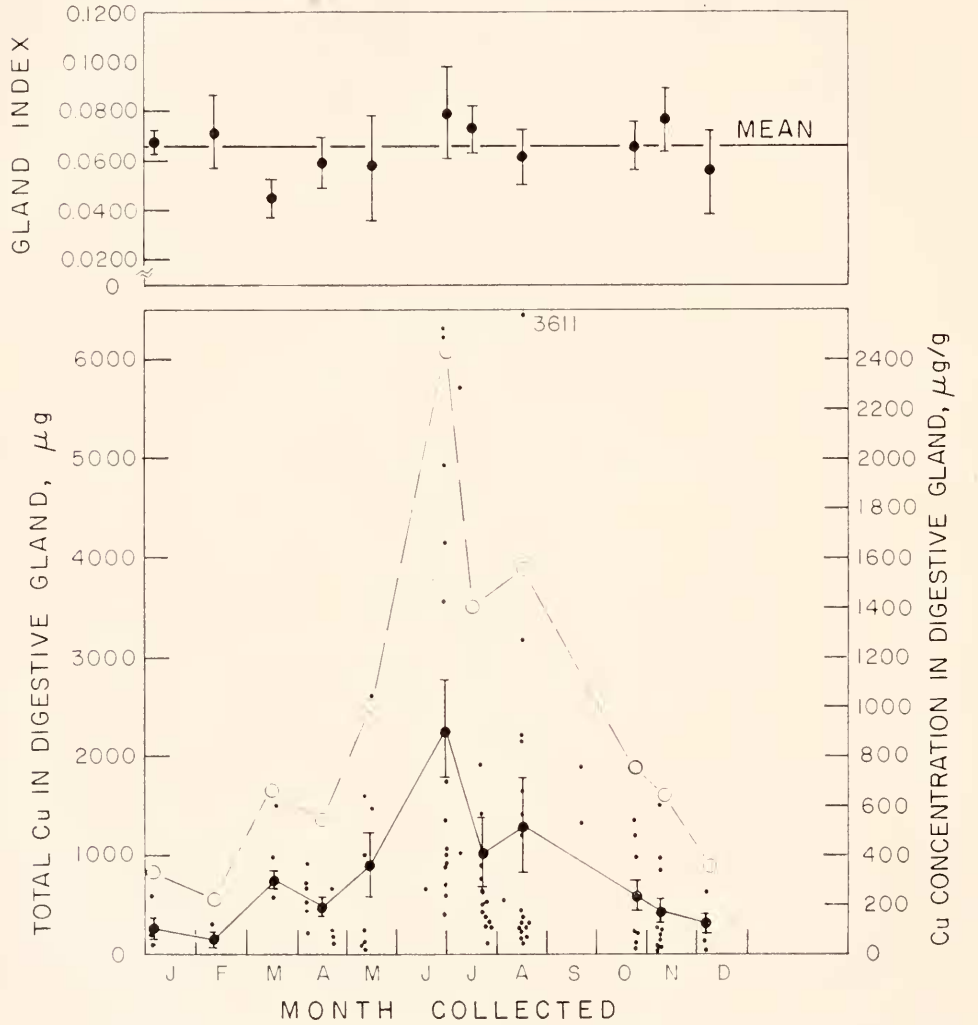

FIGURE 2. Annual changes in digestive gland index and digestive gland copper in *Busycon*. Digestive gland index (fresh weight digestive gland/fresh weight whole soft tissues): closed circles, average index for month ± two S.E.M.; open circles, monthly average total copper in digestive gland of 120-gram whelk (see text); closed circles, average copper concentration (μg/g) for month ± one S.E.M.; points represent concentrations in individual digestive glands.

well-developed penis or nidamental gland) are found in the spring. Relative weights (or "indices") of the other tissues did not show seasonal changes (*e.g.*, Fig. 2) except for a possible fall increase in the kidney (Fig. 4).

The copper concentrations in the tissues of *Busycon* were characterized by a high degree of individual variation, even in animals captured at the same time. This variability is illustrated in the plots of individual concentrations of copper in the digestive gland and blood (Figs. 2 and 3) and by the standard errors of the monthly means plotted for the gonad and kidney (Figs. 1 and 4). Despite the striking variability, clear seasonal patterns of copper concentration are evident for most tissues analyzed. All concentrations are reported in terms of μg Cu/g fresh weight. Total copper in each individual tissue of each whelk was normalized to that in an hypothetical "average whelk," using as a multiplication factor the ratio of 120 grams to the actual whole soft tissue weight. Monthly averages of these totals are plotted for the gonad, digestive gland, and kidney (Figs. 1, 2, and 4).

The digestive gland (= "hepatopancreas" or "liver") and gut showed the highest copper concentrations of all tissues analyzed, with that of the digestive

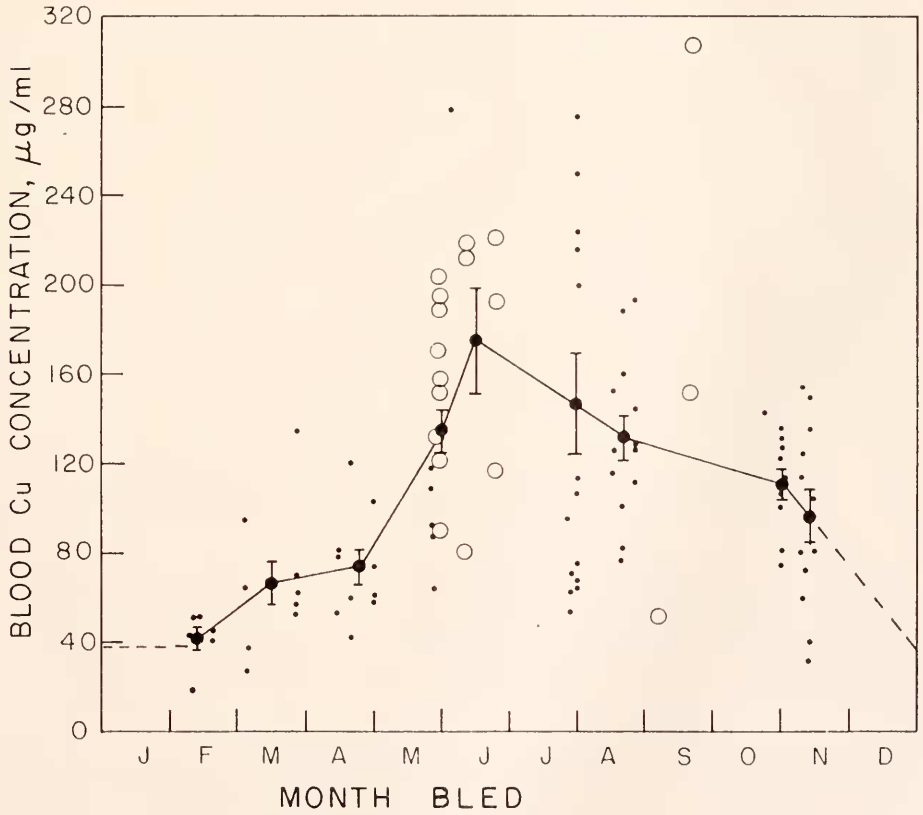

FIGURE 3. Annual changes in copper concentration of the blood of *Busycon;* individual whelks bled by slashing foot; open circles, individual whelks bled by withdrawing blood through operculum; closed circles, average copper concentration for month ± one S.E.M.

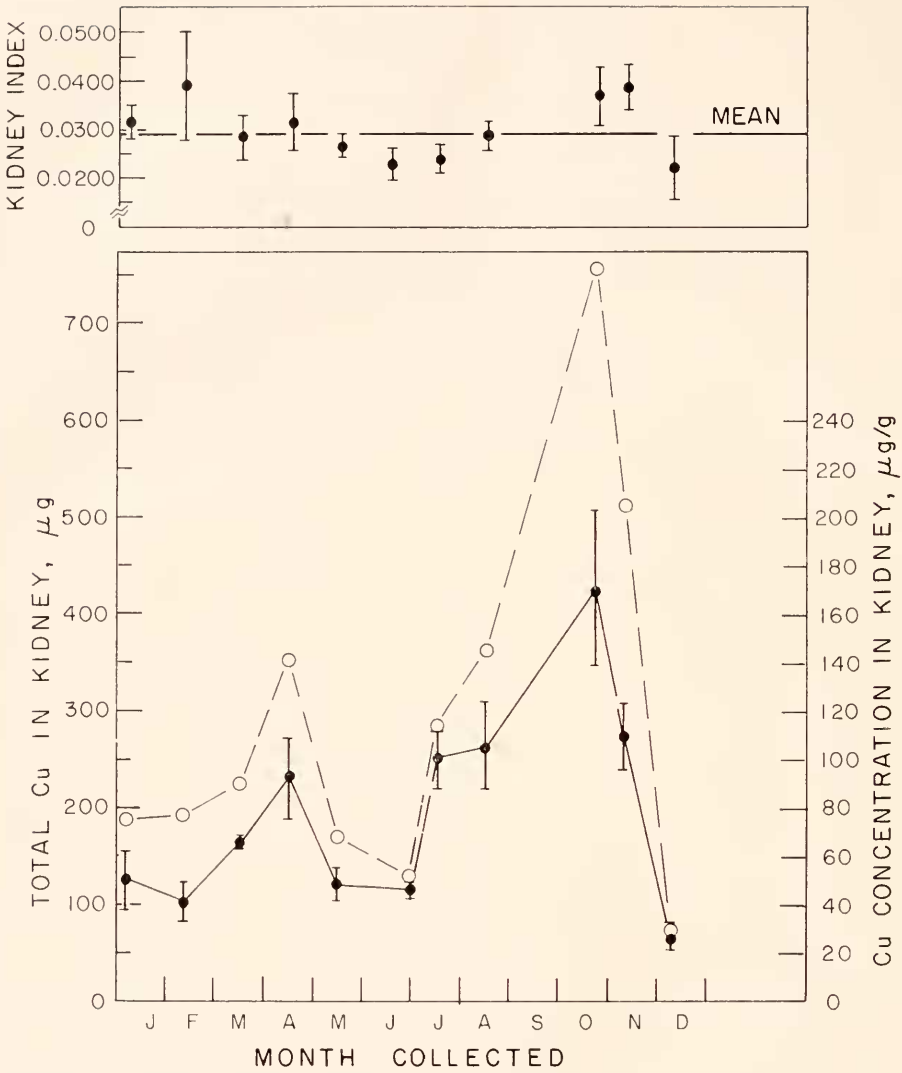

FIGURE 4. Annual changes in kidney index and kidney copper in *Busycon*. Kidney index (fresh weight kidney/fresh weight whole soft tissues); closed circles, average index for month ± two S.E.M.; open circles, monthly average total copper in kidney of 120-gram whelk (see text); closed circles average copper concentration (μg/g) for month ± one S.E.M.

gland being generally about twice that of the gut. The extreme values for the digestive gland were a low of 14 μg/g in a whelk collected in December and a high of 3611 μg/g in one collected in August. Values reported by Vinogradov (1953) for the digestive gland of other gastropods are low in comparison to the concentrations found during most of the year in *Busycon: Helix pomatia*—19 μg/g, *Purpura lapillus*—14 μg/g, and *Patella vulgata*—20 μg/g. Townsley's (1954)

unpublished determinations of the copper concentration of small subsamples of the gut and digestive gland of *Busycon* fell within the range presented here, although he separated the gland into a "liver" and "pancreas" portion and found that the gut had a higher copper concentration than either of these portions.

In the large number of digestive glands analyzed in the present study, there was a seasonal trend, with low copper concentrations in the winter and early spring (monthly means of 65–120 μg/g), higher values in May and June (when the whelks emerge from winter hibernation), and a maximum (marked by considerable variability) in the summer (monthly means of 400–900 μg/g); copper concentrations were found to decrease again in the fall. The digestive gland index remained approximately the same throughout the year (Fig. 2); thus the 5- to 10-fold difference between midwinter and midsummer concentrations is reflected in changes in the total amount of copper in the digestive gland (Fig. 2). These changes were paralleled by changes in gut copper concentration, and the increased levels of copper presumably occurred when the whelks began to feed in the spring.

The seasonal pattern was quite different in the gonad, the organ next highest in copper concentration (Fig. 1). Here the copper concentration increased in early spring, reached a maximum in April, and steadily decreased from May to a low in midsummer, remaining low and fairly constant until the March increase. Because of concomitant increases in the gonad index, however, total copper in the organ actually increased 6-fold during the summer as spawning approached, even while the copper concentration decreased. By late fall, total copper had again decreased to winter levels.

In the blood of *Busycon*, copper is present as part of the metallo-protein, hemocyanin. For blood samples from 104 whelks, blood copper showed a linear relation to blood protein ($r = 0.97$): Cu (mg/ml) = 0.00235 protein (mg/ml)—0.0064. The copper averaged 0.235% (by weight) of the total protein in centrifuged blood; this is close to the concentration of 0.245% in purified *Busycon* hemocyanin (Hernler and Phillipi, 1933, cited in Redfield, 1934). This close agreement and small negative y-intercept suggest that only a small amount of the protein in the blood is not associated with copper (averaging 2.8 mg/ml); the presence of only this small quantity of non-hemocyanin protein is consistent with Ghiretti's (1966) statement that 90% of total blood protein in *Busycon* is hemocyanin. The consistent linear relationship between copper and protein and the good agreement with the literature value for the percentage of copper in *Busycon* hemocyanin provide additional evidence for the accuracy of copper determinations in the present study.

The relation between blood copper and protein did not change over the year; but their concentration showed great variability (Fig. 3), with extreme values of 19 μg Cu/ml (in February) and 316 μg Cu/ml (in July). This 17-fold variation in copper (and hemocyanin) concentration is much greater than the two-fold range in *Busycon* hemocyanin concentration reported by Redfield, Coolidge, and Hurd (1926) and Townsley (1954), perhaps because these investigators each sampled at a single season of the year. The large variation found even among whelks bled at the same time in our study was not correlated with sex or body weight.

The concentration of blood copper (and hemocyanin) rose and fell at roughly the same times of year as in the digestive gland and gut (although the copper

concentration of the blood and digestive gland showed no direct relationship in individual whelks). In February, the average was 42 μg Cu/ml, rising sharply at the time of emergence from hibernation in May and June to a maximum of 175 μg/ml (Fig. 3), or about four times the winter level. There was a gradual decrease in concentration in the fall.

The seasonal pattern of copper concentration and total copper in the kidney (Fig. 4) was different from that of the other copper-rich tissues of the visceral mass. This organ was for most of the year comparable to or lower in copper concentration than the blood, but in the late summer and early fall, when the blood, digestive gland, and gut were decreasing in copper concentration, the kidney reached a maximum concentration (averaging 171 μg/g), exceeding the copper concentration of the blood. Total copper increased even more sharply, because of a larger kidney index. It then decreased to low values in the winter and spring. In a few whelks dissected in late summer and fall, the kidneys were noted to be dark blue instead of their usual brown color; on analysis these kidneys were found to have unusually high copper concentrations, and total copper (amounting to 1090 μg, 1895 μg, and 1137 μg in three kidneys) even exceeded that of the digestive gland. This pattern suggests that perhaps the kidney may have a role in excreting some of the copper accumulated over the summer. That the blood has not yet at this time decreased to its average winter copper concentration is consistent with a role in transporting tissue copper to the kidney for excretion.

Several other tissues were routinely analyzed—a sample of foot muscle, the whole osphradium, and the whole gill. The first two were generally very low year round and showed probably only random variation, the foot muscle averaging less than 20 μg/g for every month sampled, and the osphradium always about 40 μg/g or less. The gill, an organ richly supplied with blood, followed a pattern of copper concentration that paralleled that of the blood, ranging from an average of 15–30 μg/g in the winter to 70–80 μg/g in the summer. Probably the blood present in all three of these tissues accounted for the bulk of the copper.

Copper concentration of entire soft tissues

Six whelks captured over the course of a summer (May 15–Sept. 20) were analyzed for the copper content in total body tissues (Table I). The copper concentration ranged from 58–116 μg/g, with a mean of 76 μg/g; this is at the high end of the range of values collected by Vinogradov (1953) for other marine gastropods: *Haliotis cracherodii*—0.8 μg/g, *Littorina*—4 μg/g, *Murex*—21 μg/g, and *Buccinum*—78.5 μg/g. The total body copper concentrations in three species that can be calculated from the data of Segar *et al.* (1971) are also lower than found here in *Busycon: Patella vulgata*—1.4 μg/g, *Buccinum undatum*—40 μg/g, and *Crepidula fornicata*—40.5 μg/g. The digestive gland contained the bulk of the tissue copper in these summer whelks, ranging from 47–82% of the total, with an average of 60%.

In the whelks analyzed there was no apparent relationship between the size of the animal and its copper concentration. For the copper determinations on both these animals in which entire soft tissues were analyzed and also those in which only individual organs were analyzed, some whelks taken in the summer were dissected within a few days of capture; others were kept without feeding up to 8 weeks

before dissection. There was thus considerable variation in the period which had elapsed since the last possible feeding, but this had no apparent effect on the copper content of the whole whelk, digestive gland, or blood, or on the fraction of copper in the digestive gland. This suggests that, even if the diet is the main source of copper, frequent feeding is not necessary to maintain the high body copper concentrations found, and, in addition, that copper stored in the digestive gland is not rapidly depleted. Apparently, once copper has been accumulated in the late spring or early summer, it is not quickly turned over or excreted.

Discussion

The high degree of variability in copper concentration found in *Busycon* tissues, particularly the digestive gland and blood, is less than was found for *Haliotis* blood (Pilson, 1965) but is similar to that noted by Rocca (1969) for the hepatopancreas of *Octopus vulgaris*. He found no significant correlation between the copper concentration of the digestive gland and the season when the animals were killed and suggested that the unusually high variability might be due to its role

Table 1

Copper content of entire soft tissues of whelks and percentage Cu in digestive gland

Whelk no.	Sex	Tissue wt, g	Month captured	Days starved	Total Cu μg	μg Cu / g fresh wt	% of Cu in digestive gland
8	♂	31	May	55	1,801	58	47
9	♀	173	July	18	11,582	67	55
11	♀	220	Aug	9	15,484	70	70
16	♀	177	July	59	20,579	116	82
20	♂	54	Sept	33	3,618	67	52
21	♂	97	Sept	33	7,238	81	57
$\bar{x}$						76	60

in copper absorption and storage in this animal. Weischer (1965) found seasonal variation as well as pronounced individual scatter in the blood and hepatopancreas copper concentrations of *Helix pomatia*, the terrestrial pulmonate snail; that of the hepatopancreas was highest in the summer, when the copper concentration of the blood was very low. In the fall, just before hibernation, the copper concentration in the blood climbed sharply and that in the hepatopancreas dropped. The blood copper concentration reached a maximum in January and was gradually reduced in the early summer. Weischer concluded that copper from the degradation of hemocyanin was stored in the hepatopancreas during the summer and used in autumn for renewed hemocyanin synthesis. The role, if any, for the very large reservoir of copper in the digestive gland of *Busycon* remains unknown.

The findings of both Weischer's and the present study tend to contradict Ghiretti's (1966) statement that in mollusks "during the life span of an animal the concentration of hemocyanin seems to remain fairly constant under normal physiological conditions" (page 234). In *Busycon*, unlike *Helix*, the copper concentrations in the digestive gland and blood do not vary reciprocally over the year;

instead, both tend to rise with the onset of feeding in early summer and to decrease in the fall. The differences in cycles of copper concentration in the tissues of the two species of snail may reflect differences in copper availability in their environments, as well as differences in their annual cycles of spawning and activity, and mode of hibernation. In *Helix*, blood copper is highest during hibernation; in *Busycon*, it is lowest during this period. *Busycon* has abundant copper available in the diet and lives bathed in a copper-containing solution: *Helix* has more limited access to the metal and so perhaps exercises greater control over storage and reuse of its supply. From neither of these studies can firm conclusions be drawn as to the primary function of hemocyanin in the snails; the evidence of seasonal variation could be used to support either a respiratory role or a role as a food (energy) reservoir, although the extreme variability argues against a primarily respiratory function (Pilson, 1965). Both studies underline the potential errors inherent in reports of observations of metal concentrations from small numbers of individual invertebrates without regard to season or physiological state of the animals.

We wish to thank Richard Sisson, of the Rhode Island Department of Natural Resources, and Stanley Spink for providing specimens of *Busycon*. Robert Duce kindly made available his Perkin-Elmer 303 atomic absorption spectrophotometer. Peter Betzer helped with copper analyses and with preparation of the manuscript. This work was done at the Graduate School of Oceanography of the University of Rhode Island while one of us (S. B. B.) was supported by a National Science Foundation Graduate Fellowship.

Summary

Individual tissues of approximately 100 whelks were analyzed for copper content. Tissue copper concentrations showed a high degree of individual variation, but seasonal trends were evident. In the digestive gland, the organ with the highest copper concentration, the mean monthly concentrations were about 5 to 10 times higher in the summer (400–900 μg Cu/g fresh weight) than in the winter (65–120 μg Cu/g). Gut concentrations followed the same seasonal trend. The copper concentration in the gonad increased in the early spring and then decreased in the late spring and summer to low fall and winter values. The total copper in the gonad increased 6-fold during the spring and summer, however, because of large increases in size as late summer spawning approached. The total copper in the gonad dropped off after the spawning season. Monthly average blood copper and protein concentrations followed the same seasonal pattern as the digestive gland and gut, with copper ranging from a monthly average of 42 μg/ml in February to 175 μg/ml in June. Nearly all the blood protein present was hemocyanin, and there was no apparent seasonal change in the ratio of copper:protein. The copper concentration of the kidney was similar to that of the blood during most of the year, but in late summer and fall it increased to a maximum (171 μg/g in Oct.), while that of the blood and other tissues was decreasing. Whelks in Narragansett Bay usually begin to feed in late May or early June; the general early summer increases in the copper concentration of most tissues correlated with this commencement of feeding.

LITERATURE CITED

Betzer, S. B., 1972. Copper metabolism, copper toxicity, and a review of the function of hemocyanin in *Busycon canaliculatum* L. *Ph.D. dissertation, University of Rhode Island,* 133 pp.

Diehl, H., and G. F. Smith, 1958. *The Copper Reagents; Cuproine, Neocuproine, and Bathocuproine.* The G. F. Smith Chemical Company, Columbus, Ohio, 48 pp.

Ghiretti, F., 1966. Molluscan hemocyanins. Pages 233–248 in K. M. Wilbur and C. M. Yonge, Eds., *Physiology of the Mollusca Vol. 2.* Academic Press, New York.

Goldberg, E. D., 1965. Minor elements in sea water. Pages 163–196 in J. P. Riley and G. Skirrow, Eds., *Chemical Oceanography Vol. 1.* Academic Press, New York.

Layne, E., 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Pages 233–245 in S. P. Colowick and N. O. Kaplan, Eds., *Methods in Enzymology Vol. 2.* Academic Press, New York.

Marks, G. W., 1938. The copper content and copper tolerance of some species of mollusks of the southern California coast. *Biol. Bull.,* **75**: 224–237.

Pilson, M. E. Q., 1965. Variation of hemocyanin concentration in the blood of four species of *Haliotis. Biol. Bull.,* **128**: 459–472.

Redfield, A. C., 1934. The haemocyanins. *Biol. Rev. Cambridge Phil. Soc.,* **9**: 175–212.

Redfield, A. C., T. Coolidge, and A. L. Hurd, 1926. The transport of oxygen and carbon dioxide by some bloods containing hemocyanin. *J. Biol. Chem.,* **69**: 475–509.

Riley, J. P., and D. A. Segar, 1970. The distribution of the major and some minor elements in marine animals. I. Echinoderms and coelenterates. *J. Mar. Biol. Ass. U.K.,* **50**: 721–730.

Riley, J. P., and P. Sinhaseni, 1958. The determination of copper in sea water, silicate rocks, and biological materials. *Analyst,* **58**: 299–304.

Rocca, E., 1969. Copper distribution in *Octopus vulgaris* Lam. hepatopancreas. *Comp. Biochem. Physiol.,* **28**: 67–82.

Segar, D. A., J. D. Collins, and J. P. Riley, 1971. The distribution of the major and some minor elements in marine animals. II. Molluscs. *J. Mar. Biol. Ass. U.K.,* **51**: 131–136.

Townsley, S. J., 1954. Studies on copper in mollusks, with particular reference to *Busycon canaliculatum* Linnaeus. *Ph.D. dissertation, Yale University,* 126 pp.

Vinogradov, A. P., 1953. *The Elementary Chemical Composition of Marine Organisms.* Memoir II, Sears Foundation for Marine Research, 647 pp.

Weischer, M. L., 1965. Stoffwechselphysiologische Untersuchungen zur Bedeutung des Haemocyanins bei *Helix pomatia* L. *Zoologische Beiträge (Berlin),* **11**: 517–540.